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Leucine-glycine and carnosine dipeptides prevent diabetes induced by multiple low-dose streptozotocin in experimental model of adult mice

Short title: Leucine-glycine and carnosine prevent

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ABSTRACT

Aims/Introduction: Peptides are considered as quasi-hormones and effective molecules for regulation of the cells function and metabolic disorders prevention. Di- and tripeptides with the ability to gastrointestinal absorption have been proposed to prevent diabetes progression.

Materials and Methods: Small peptides with different sequences of specific amino acids were synthesized based on a solid phase peptide synthesis (SPPS) protocol as well as carnosine (A) and glutathione (B) were examined for the prevention of diabetes induced by multiple low-dose of streptozotocin (MLDS) in mice.

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Results: The peptides A, Leu-Gly (D) and Pro-Pro (F) exhibited a preventive effects on blood glucose elevation and impairment of the signaling and performance of beta cells. The beta cells function assessed by immunofluorescence and blood glucose level in mice exposed to diabetes treated by the peptides A and D was similar to the normal mice. The peptide D prevented from body weight loss caused by diabetes induction. The use of D and A peptides dramatically prevents the incidence of disruption in beta cells signaling by maintaining the natural balance of intracellular Akt-2 and cAMP.

Conclusions: The results proved that peptide D (Leu-Gly) named Hannaneh inhibits the body weight loss caused by diabetes induction. The Hannaneh and carnosine dipeptides with preservation of normal beta cell signalling and anti DPP-4 activity were prevented from increasing the blood glucose in mice at risk of diabetes. These dipeptides may be regarded as the pharmaceutical agents for the prevention of diabetes.

Keywords: Beta cell, Glucose, peptide,

INTRODUCTION

Recent years have witnessed a peptides revolution as a result of newly discovered influences of peptides on different physiological systems. Small peptides are defined as quasi-hormones and pharmaceutical agents; these bioactive molecules can modulate the cells physiological functions¹. Bioactive small peptides can be absorbed from the intestine directly into bloodstream via the PepT1 H⁺/peptide co-transporter². After enteric absorption, the pancreas is one of the important organs as it is exposed to high concentrations of absorbed bioactive peptides. Beta cells in the pancreas are one of the major cellular targets for bioactive peptides^{3, 4}.

Beta cells are very sensitive to apoptotic damage induced by oxidative and inflammatory induced compounds. Streptozotocin (STZ) as a chemical toxin binds to the GLU2 receptors on beta cells^{5, 6}. Administration of multiple low-dose streptozotocin (MLDS) induces distortion of the beta cells in conjunction with mononuclear cell infiltration and apoptosis by caspases enzymes, DNA mutilation, nitric oxide production, and free radical generation^{7, 8}. It is known that the STZ model of diabetes simulates the complications of human diabetes. Given the established similarities of some structural, functional, and biochemical abnormalities to human disease, it is an appropriate model for assessing the mechanism of diabetes⁷. Although there is disagreement among researchers, the majority agree that MLDS generated mild hyperglycemia (200-400 mg/dl) in experimental mice gives insulin secretion that is similar to human T2D⁹. However, there are conflicting reports indicating that administration of MLDS in first days generates T2D and in progression produces T1D^{10, 11}.

GLP-1, a major incretin hormone released in response to nutrient intake and bloodstream amino acids and peptides, contributes over 50% of postprandial glucose-stimulated insulin release and plays important roles in insulin secretion and maintaining normal glucose regulation^{12, 13}. The durability of this hormone is transient because it is rapidly cleared by the kidney and inactivated by cleavage at the N-terminal by dipeptidyl peptidase-4 (DPP-4) enzyme¹⁴. Therefore, it is important for T2D therapy to develop a potent DPP-4 inhibitor, GLP-1 stimulator, and direct beta cell-signaling activator from natural sources such as peptides¹⁵. The receptors for GLP-1 are located in the pancreas of humans and mice. Previous studies have demonstrated the proliferative and antiapoptotic effects of GLP-1, leading to the expansion of the mass of beta cells and improved glucose homeostasis in T2D^{14, 16}. The serine/threonine-specific protein kinase Akt-2, also known as protein kinase B (PKB), intracellular enzyme for beta cells, plays an important role in signal transduction downstream of the insulin receptors. Activation of Akt-2 signaling in mice constitutively overexpressing

activated Akt-2 and cyclic AMP (cAMP) as the second messenger for regulation of insulin exocytosis in beta-cells resulted in increased islet mass, largely owing to neogenesis, proliferation of beta cells, and improved glucose tolerance. Therefore, Akt-2 might represent a potential target to improve beta cell proliferation and survival^{18, 19}. GLP-1 may activate Akt-2 signaling of the cAMP-dependent pathways in beta cells. cAMP signaling is one main factor in transducing GLP-1-mediated activities in beta cells. On the other hand, GLP-1 has been shown to improve both beta cell proliferation and survival via cAMP-dependent stimulation of the cAMP in mice^{18, 19}.

One of the main causes of diabetes development is linked to oxidation phenomenon. Therefore, antioxidative peptides may exert antidiabetic effects, particularly in the prediabetes stage^{20, 21}. Some histidine (His)-containing peptides, particularly the dipeptide known such as carnosine (β -alanine-L-histidine), exhibit antioxidant activity^{20, 22}. The tripeptide glutathione exists in natural tissues and is important in the regulation of the redox state and protection of cells from oxidative damage by the disposal of free radicals, which have a pathogenic effect in the chronic complications of diabetes²³. Hydrophobic amino acids such as proline (Pro) and leucine (Leu), especially in the C- and N-terminals of peptides, have antioxidative effects²⁰. Pro is an essential amino acid in regulating gene expression, cell signaling, and antioxidative responses. This amino acid also modulates protein–protein interactions, thus playing important roles in many cell-signaling pathways. Leu is an important branched-chain amino acid owing to its most popular benefit in the metabolism. It activates the mTOR and cAMP pathways, leading to increased protein synthesis, specifically insulin, by beta cells (as an insulin production stimulator) and prevents mitochondrial gene mutations (one of the causes of diabetes and obesity). Leu and its small peptides are known to stimulate insulin secretion from pancreatic beta cells by serving as metabolic fuel and regulating cell metabolism. Furthermore, Leu peptides regulate the expression of key

metabolic genes in beta cells as a new opportunity for prevention of islet dysfunction and diabetes^{23, 24}. The peptides of the glyproline family exhibit various bioactivities and the linear glyproline peptides are relatively stable ($t_{1/2}$ of Pro-Gly-Pro is more than 24 h)^{24-26, 28}.

The objective of the present study was to investigate the antidiabetic effects of di- and tri-peptides with different sequences consisting of three specific amino acids, including Pro, Gly, and Leu.

MATERIALS AND METHODS

Peptides synthesis

Experimental peptides were synthesized using a classical glass reaction vessel following the Fmoc solid-phase peptide synthesis protocol^{29, 31}. The 2-CTC resin (specific for Pro and Gly peptides to prevent diketopiperazine formation) was swelled in dry DMF (this resin is extremely moisture-sensitive) for 1 h. Then the DMF was filtered under vacuum. The first amino acid at the C-terminal of the peptide was coupled in the presence of TBTU and DIPEA in dry DMF by mixing with nitrogen gas for 2 h. After the completion of the coupling reaction (which was confirmed by Kaiser test³⁰ for Leu and Gly and isatin test for Pro²⁹), the solution was filtered off and then the resin coupled with the first amino acid was washed with DMF (3×2 mL) and DCM (3×2 mL). In order to ensure that no unreacted sites remained on the resin, the resin was end-capped (twice) using HPLC-grade methanol MeOH. The N-terminal Fmoc deprotection was performed using piperidine in DMF (20% v/v, 10 mL, and 2×30 min). The resin was washed with DMF (3×2 mL) and DCM (3×2 mL). The subsequently activated amino acids were coupled in a similar manner. The resin was washed (three times) with DMF and DCM, sequentially, at the end of all stages. After obtaining the required peptide sequence, the resin-peptide was dried under vacuum for 24 h. Fresh cleavage cocktail, composed of 5% TFA in DCM, was added to the resin-peptide. The

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mixture was shaken at room temperature for 2 h then the resin was collected by filtration. The isolated peptide was washed with another 2 mL of cleavage cocktail. The combined filtrates containing 5% TFA in DCM and the peptides were evaporated to a minimum volume using a nitrogen evaporator. The resulting solution was lyophilized for 24 h. Purification of peptides was carried by analytical RP-HPLC using a Zorbax 300 SB-C₈ narrow bore column with a linear AB gradient (1% acetonitrile/min) at a flowrate of 0.25 mL/min, where eluent A was 0.2% *aq.* TFA, pH 2, and eluent B was 0.2% TFA in acetonitrile. Eventually, about 200 mg of each di- and tri-peptide was obtained. The chemical structures of the seven most effective peptides that were produced and studied in the present research are shown in Figure 1.

Animals, experimental design and tested peptides

Male BALB/c mice (age 9-10 weeks) were purchased from the Pasteur Institute of Tehran (Iran) and were housed in a controlled environment in a specific pathogen-free animal facility at 22°C under a 12: 12 h light: dark cycle at the Drug Applied Research Center, Tabriz University of Medical Sciences. The normal diet and water were offered *ad libitum*. Following animal adaptation to the environment for 1 week, all mice were weighed, labelled, and allocated to treatment groups on the basis of the average weight (35.4 ± 0.3 g) (10 male mice per group). After a week of re-adaptation, the experiment was started. On the first day of the experiment, simultaneous injection of STZ and peptides was commenced. Diabetes was induced by MLDS injection on the left side of the intraperitoneal (IP) space. For this purpose, The STZ (S0130/Sigma) was dissolved in 0.1 M citrate buffer (pH 4.5) and injected within 20 min of preparation at a dose of 50 mg/kg/day for 5 consecutive days. Coinciding with the STZ injection, each of the peptides was injected on the right side every day (peptides dissolved in dimethyl sulfoxide, DMSO, 3% in PBS and injected at 20 mg/kg of body weight/day). Briefly, the experimental treatments were as follows: in the control group

(CON), mice received bilateral IP injections of citrate buffer without STZ (left side) and PBS without peptide (right side). In the group exposed to diabetes (DI), the mice received bilateral IP injections of STZ (left side) and PBS without peptide (right side). The third group (DM) was mice exposed to diabetes and 3% DMSO that received bilateral IP injections of STZ (left side) and PBS + 3% DMSO without peptide (right side). The other groups of mice received peptides and exposed to diabetes where each group received one of the peptides dissolved in 3% DMSO in the PBS, the mice with bilateral IP injections of STZ (left side) and one of the peptides + 3% DMSO in PBS (right side) (seven groups for the seven reported peptides from A to G). In all groups, STZ was injected from 1 to 5 days and peptides were injected from 1 to 12 days continuously. The body weight of each of the labelled mice was recorded at three time points (1st, 6th, and 12th days of the experiment).

In this study, 15 peptides including Leu-Pro-Pro, Leu-Pro, Leu-Leu, Leu-Leu-Gly, Leu-Gly, Leu-Leu-Leu; Pro-Gly-Pro, Pro-Pro, Pro-Leu-Gly, Pro-Gly, Pro-Pro-Pro; Gly-Gly, Gly-Gly-Gly, Gly-Pro-Gly and Gly-Leu-Gly were prepared. These peptides were tested in 3 stages under the same environmental and nutritional conditions, and the same mice species. In each of the 3 experiments there were three control groups (CON, DI and DM) and some of these 15 peptides were tested with limited number of animals. Based on our preliminary screening experiments in the final stage (main experiment), a re-test with 5 peptides including Leu-Pro-Pro, Leu-Gly, Pro-Gly-Pro, Pro-Pro, and Pro-Gly which exhibited promising anti-diabetic effects along with the previously known effective peptides i.e. Glu-Cys-Gly (glutathione) and Ala-His (carnosine) were tested.

Glucometry, insulin, GLP-1, and DPP-4 Assay

On the even days of the experiment (days 2, 4, 6, 8, 10, and 12), blood samples were obtained from the tail vein of each mouse in the fed state and glucose was measured using a glucometer (GALA, TD-4277, 2016 approved by the FDA) between 9:00 and 11:00 am. Mice were considered diabetic when their fed blood glucose level was >200 mg/dl. Furthermore, the other blood samples (fed state) were taken from the orbital sinus at days 6 (9:00 am) and 12 (11:00 am) for measurement of insulin, GLP-1, and DPP-4 at a maximum of 30 min after peptide injection. For this purpose, obtained sera were measured by ELISA kits according to the manufacturer's instructions (AWAKNESS Technology Inc., USA), including an ELISA microplate reader (Stat Fax 2100) and an automatic ELISA plate washer (Stat Fax 2006) for mouse insulin (Sigma-Aldrich, USA), mouse GLP-1 (Eastbiopharm Inc., China), and mouse DPP-1 (Eastbiopharm Inc., China).

Dissection and isolation of mice pancreatic islets

At the end of the experiment, the pancreases were dissected from anesthetized (by ketamine and xylazine) mice. Islets were isolated from the pancreas based on the described protocol for collagenase digestion³³. After being washed in Krebs–Ringer bicarbonate buffer solution with 0.2% BSA, the islets were digested with 0.025% trypsin (Life Technologies) for 5 min at 37°C and passed through a 400-µm wire mesh. The cells were then plated on coverslips that were coated with poly-L-lysine and maintained at 37°C in a 5% CO₂ incubator for 24 h^{32, 33}.

Immunofluorescence imaging

The dissociated islet cells were fixed in 7% paraformaldehyde for 15 min and washed with 0.1 M PBS with 0.3% Triton X-100 for 30 min at 37°C. After incubation in 5% BSA and 0.15% Triton X-100 blocking solution for 1 h, the cells were incubated by primary antibodies

for 1 h at 4°C, washed with PBS, and incubated again with the appropriate fluorochrome-conjugated antibodies for 1 h. Antibodies included a guinea pig anti-insulin antibody (Abcam, UK), a mouse anti-glucagon antibody (Abcam, UK), and a mouse anti-somatostatin antibody (Abcam, UK). After being thoroughly washed with PBS, immunofluorescence images of the islets were obtained based on minimal background under a total internal reflection fluorescence microscope because high background fluorescence may occur as a result of exocrine contamination³³.

Akt-2 and cAMP assay

Isolated islets were used to assay Akt-2 and cAMP of beta cells. The extracted biomass were diluted in 10×volume of 0.1 M HCl, homogenized, and centrifuged at 10000 rpm for 5 min. After incubation, the surface liquid used for cAMP lysates was measured using the direct Cyclic AMP EIA kit (Assay Designs) and then Akt2 (AKT2 ELISA Kit, My Biosource, Inc. California, USA) according to the manufacturer's instructions^{33, 45}.

Statistical analyses

All variables were checked for normality using the Kolmogorov-Smirnov (K-S) test using SPSS, version 19.0 (SPSS Inc., Chicago, IL, USA). K-S test revealed that all variables were normally distributed. Data were analyzed by one-way (glucose data) or two-way (hormone data) GLM procedure ANOVA using SAS for Windows release 9.2 (SAS Institute Inc., Cary, NC, USA). Data are reported as the mean and standard deviation (SD). The differences between means were studied by Duncan's multiple-range test where $P < 0.05$ were reported as statistically significant.

RESULTS

Table 1 presents the mean plasma glucose levels for the six timepoints of the experimental period and statistically compares these values between treatments. The glucometry of the second day of intervention demonstrates that in the exposed to diabetes mice (DI), the glucose level was significantly elevated compared to the control (CON) group (111.6 versus 94.6 mg/dl) ($P<0.01$). The mice received peptide E exhibited smaller amounts of blood glucose level than those DI and CON groups ($P<0.01$). On the fourth day of the trial, the blood glucose level was higher than that of most groups in mice that were in DM and peptide D groups ($P<0.05$). Moreover, the glucose level in the mice that received peptide E was lower than all other groups ($P<0.05$). The mice received peptides A, B, C, and G exhibited lower amounts of blood glucose level compared to the DI group at the 6th day. In addition to Table 1, the charts in Figure 2 illustrate the effects of the peptides at days 10 and 12 days of the experiment. Ten days after the start of the trial and five days after MLDS protocol completion, the blood glucose was significantly higher in mice of the DI and DM groups compared to the CON group (174.8 and 177.3 versus 111.7 mg/dl) (Figure 2, chart L). Two diabetes-exposed groups treated by peptides A and D had blood glucose levels similar to those of the CON group (116.4 and 112.0 versus 111.7 mg/dl). The mice received other peptides exhibited intermediate amounts of blood glucose level between the CON and DI groups. In other words, peptides A and D prevented hyperglycemia under the influence of STZ injection at this stage (Figure 2, chart L). On the 12th day of the experiment, the mice of DI and DM groups were completely diabetic (glucose levels 279.3 and 219.1 mg/dl, respectively). Also, the results (Table 1 and Figure 2, chart R) showed that the difference between blood glucose levels between the DI and DM groups was significant at the end of the experiment ($P<0.01$). The mice received DMSO exhibited smaller amounts of blood glucose than DI group. The blood glucose levels of mice that received peptides A, D, E, and F were

statistically similar to those of the CON mice at the end of the experiment. It is clear that the differences between the glucose levels for the CON mice in the six time stages of glucometry were not significant (respectively, 94.6, 107.4, 120.3, 99.2, 111.7 and 118.7 mg/dl). The blood glucose levels in the DI mice increased with a steep slope during the six stages. Similar result was obtained for the DM group that received 3% DMSO in PBS, So that, the mice in this group (DM) were diabetic by the 12th day of the trial (219.1 mg/dl). The differences between the means of blood glucose levels at the six glucometry times were not significant in mice received peptides A, D, and F ($P>0.05$). This means that the use of any of these three peptides dramatically reduced the incidence of diabetes. However, the other peptides also effectively prevented the excessive increase of glucose levels until the 10th day of the trial. The results showed that the blood glucose levels in mice that received any of the peptides did not exceed 200 mg/dl (diabetic level), even on the last day.

The body weight of the mice was determined at three time points (days 1, 6, and 12) and the results are shown in Figure 3. Owing to the initial control of the mice weight at the start of the experiment, the weight differences were not significant at the first day. On the 6th day of the experiment the differences in their mean body weight between treatments were significant ($P<0.05$). The induction of diabetes caused a reduction in the body weight in the DI mice, whereas in the CON group the body weight was significantly elevated in ascending order from days 1 to days 12 (Figure 3). The injection of all peptides and also DMSO prevented the body weight loss, except peptides A, B, and G. Peptide D was the most efficient at preventing body weight loss.

As shown in Figure 4, on the 6th day of the experiment the blood insulin levels in the CON and DI groups were not statistically significant (5 and 5.1 mIU/L). While, the insulin level in the mice that received peptides E and G (3.7 and 3.0 mIU/L, respectively) especially peptides A and B (2.04 and 2.80 mIU/L, respectively) exhibited lower levels. Data obtained on the

12th day for insulin level showed that, in contrast to the 6th day, the insulin levels of the CON, DI, and DM mice were lower (2.9, 2.8, and 2.5 mIU/L, respectively) than insulin levels in mice treated by peptides A, B, and D ($P<0.05$). It should be noted that as mice are nocturnal animals and eat overnight, the level of insulin in their blood is therefore higher in the early morning compared to later in the morning. This could be why there was an observed difference in insulin level for the control group for the 6th and 12th days of the experiment.

According to Table 2, the difference between the mean GLP-1 levels in the CON and DI mice was significant on the 6th day ($P<0.05$). The GLP-1 level was elevated in the DI group compared to the CON group one day after the end of the MLDS protocol. All peptides except peptides B and G prevented GLP-1 increasing in DI mice at the 6th day. However, the difference in the GLP-1 levels in the CON and DI groups was not significant on the 12th day.

Moreover, the mice received peptides D or F and the DM group mice exhibited a decline in GLP-1 amounts that is not significantly different compared to the CON and DI groups. Development of diabetes did not have an effect on DPP-4 activity. In other words, there was no significant difference between CON and DI groups at 6th and 12th days of the experiment. Although, the use of peptides, specifically A, D, and F, caused a reduction in DPP-4 activity on the 6th day of the experiment (Table 2).

Figure 5 shows that Akt-2 and cAMP levels of the isolated islets, as beta cell-signaling indicators, are affected ($P<0.05$) by treatments. The induction of diabetes (DI) has been associated with a sharp decline in both indicators of the intracellular signaling of beta cells compared to control group. Using DMSO (DM) had no effect on disorder improvement cell signaling. The mice received peptides B and E did not exhibited any effect on Akt-2 activity of the beta cells. The other peptides, particularly peptides A and D, which had a significant effect on Akt-2 and prevented intracellular signaling impairment of beta cells in the mice exposed to diabetes. The injection of the peptides C, E, and G did not have any effect on

cAMP levels. The mice received peptides A and D had a higher concentration of intracellular cAMP in their beta cells ($P<0.01$). While, mice treated with peptides B and F were affected to a certain extent. Therefore, peptides A and D prevented any interference in cell signaling by STZ-induced diabetes in mice. The immunofluorescence staining of the islets at the end of the experiment displayed a normal islet architecture, with insulin-producing beta cells in green and glucagon-producing alpha cells in red for the CON group (Figure 6). The severe destruction of endocrine cells with minimal hormonal content was observed in the islets of mice exposed to diabetes (Figure 6), similar cell-signaling disorder (Figure 5), body weight loss (Figure 3), and glucose concentration elevation (Figure 2), but not similar blood insulin levels. The A and D peptides showed preventing effects against the incidence of diabetes. The blood glucose levels were similar in mice treated with peptides A and D on the 10th and 12th days. A significant ($P<0.05$) anti-DPP-4 effect was observed on the 6th day mainly for the mice groups receiving peptides F, D, and A. As shown in Table 2, unlike the CON and DI groups, the DPP-4 enzyme activity was elevated from days 6 to 12 in all peptide groups, and particularly in the F peptide group ($P<0.05$). The injection of all the reported peptides had a significant effects on the survival and secretion activity of the endocrine cells of the islets, especially the beta cells. Of course, the role of the two peptides A and D was distinct than the others, which reaffirmed the role of these dipeptides for maintaining the normal survival and hormonal secretion of beta cells in mice exposed to STZ-induced diabetes.

DISCUSSION

Recent studies have indicated the roles of small peptides as drug active agents that can modulate the physiological functions for the prevention of diabetes^{13, 44}. The present study reports the preventive effects of small peptides in mice exposed to diabetes by hormonal (insulin and GLP-1) and enzymatic (DPP-4) communications involved in glucose

homeostasis. Small peptides with insulintropic and GLP-1 tropic effects combat obesity and T2D²⁶. *In vitro* and *in vivo* studies highlighted the potential of the peptides referred to in the *BIOPEP* database for functioning antidiabetic effects and recommended more *in vivo* studies for assessment of the type and sequences of these small active peptides³⁷. In addition, the moderate effect of DMSO as an organic solvent on diabetes suppression has already been confirmed in previous studies³⁶.

In the present study, it has been demonstrated that the produced peptides from proline, glycine and leucine especially Leu-Gly and carnosine (Ala- His) dipeptides caused a significant prevention of blood glucose elevation in mice at risk of diabetes. It has recently been reported that the tripeptides of glycine-glycine-leucine (Diapin) elevated the plasma insulin and GLP-1 levels in diabetic mice³⁰. In the present study, the peptides A and D prevented the increase of GLP-1. The results showed that the peptides A and D have anti-DPP-4 effect as diabetes incidence mechanism of MLDS as described by Lin *et al.* 2010, and blood glucose elevation 7 days after the beginning of the experiment⁸. Dietary amino acids and peptides can also induce insulin secretion via GLP-1 stimulation mechanisms. Also, some cell-permeable peptides as inhibitors of the c-Jun NH2-terminal kinase prevent cytokine-induced β -cell apoptosis. A wide range of short-length peptides have been reported to possess DPP-4 inhibitory activity and effective at stimulating insulin secretion and improving glycemic control in animal models and subjects with T2D^{33, 38}.

The results of insulin measured on the 12th day indicated that the elevated glucose levels of the diabetes exposed group has no relationship with insulin level. The elevation of blood glucose level promotes an increase demand on the pancreatic beta cells to secrete insulin to bring the glucose content in the blood to a normal level. Since this homeostasis mechanism reduces blood glucose during the prediabetes stage of diabetes development, chronic and persistent insulin resistance exposes the beta cells to an excess of glucose, thereby promoting

beta cell dysfunction, failure, and death. The beta cells respond to many nutrients in the blood circulation, including glucose, other monosaccharides, amino acids, and peptides^{19, 33}. The peptides A and D significantly caused insulin elevation compared to both control and diabetes induced groups without increasing GLP-1 and the depression of DPP-4 on the 12th day. Therefore, the therapeutic agents that can halt or prevent pancreatic β -cell failure will likely have a major impact on disease progression. The action mechanism of small peptides in target cells is conditioned by their binding to special membrane receptors and are able to influence physiological functions⁴⁴. The peptide substances have shown direct regulators or mediators of physiological processes³⁸. The important factor for a peptide action is the stability of the peptide in the bloodstream. The glyproline family were most stable in blood³⁸ and the peptides containing branched chain amino acids mainly, leucine, induced an increase in GLP-1³⁹. The endogenous peptides of containing leucine stimulated insulin secretion *in vitro* and *in vivo*⁴⁰. In a study involving several animal models of insulin resistance, leucine containing peptides displayed antidiabetic effects with a suppression in the elevation of blood glucose. It should be noted that all blood samplings and STZ administration in the present study were in fed state. MLDS under fed or fasted conditions is equally effective in experimental hyperglycemia and fasting state prior to administration of MLDS is not required. Also, fasting may be an unnecessary step in the experimental design and it is an unnecessary metabolic stress to the mice under the study⁴⁰.

A significant increase in the body weight in the control group was observed in the present experiment (Figure 3) whereas, a marked depression in the body weight was observed in diabetes exposed group. Takeda *et al.* (2012) reported that body weight increased throughout the study in non-diabetic control mice, whereas mice injected with STZ lost their body weight irrespective of the treatment⁴².

The cellular nutrition and stimulation of beta cell mitosis by these peptides can be a major factor in the prevention of the effects of STZ induced diabetes. In the present study, most of the synthesized peptides especially Leu-Gly completely prevented the body weight loss under the influence of diabetes development. Also, only dipeptide Leu-Gly had the greatest protection on both glucose elevation and body weight loss. The authors have named this peptide “Hannaneh” which means it is reliable. The presented results showed that hannaneh and carnosine can effectively prevent the rise of blood glucose elevation in the onset of diabetes. The hannaneh peptide has mild anti-DPP-4 and stimulates the insulin secretion effects probably based on the immunofluorescence imaging.

Previous studies have shown that dipeptides have multiple physiological functions, including anti-inflammation, anti-oxidant and anti-diabetic effects or increased diabetic wound healing⁴³. Furthermore, it can be postulated that these dipeptides activate AMP-protection kinase (AMPK). AMPK has been identified as an important target in the prevention and treatment of obesity and T2D⁴³. It was reported that the synthetic di- and tripeptides containing glycine such as glycine-leucine (reverse in N and C-terminal of amino acid in compare with peptide hannaneh) are a strong stimulus for GLP-1 and are involved in a two sensory mechanism (PEPT1 and calcium-sensing) receptor²¹. Also, proline-containing di- and tripeptides can cause a prophylactic antidiabetic effect in the experimental insulin-dependent diabetes mellitus in rats²⁴.

Recent findings showed that Akt-2 regulated beta cell mass by modulating proliferation, cell size and apoptosis^{33, 34}. The GLP1 promotes islet-cells survival via the second messenger cAMP. The cAMP as a main regulator in beta cell growth and survival, promotes insulin secretion and enhances the activation of the Akt-2 in response to insulin for promoting cell survival. The cAMP level in the intracellular space has important effects on ion channels and exocytosis of insulin granules.

In the present study, peptides A and D prevented endocrine beta cells injury and caused survival by reinforcing of the intracellular signaling of Akt-2 and cAMP in mice exposed to STZ oxidation and toxicity. There is a direct link between cell metabolism and the signaling cascade of beta cells for insulin secretion, cell growth, differentiation and survival of the cell. This link is important in understanding beta cells dysfunction with loss of insulin release in T2D⁴⁶. Impaired glucose-induced cAMP formation has been reported in islets from diabetic animal models with reduced insulin secretion and cAMP-elevating agents have been found to ameliorate beta cells function in diabetes. The key amino acids Leu and Pro and their small peptides play a crucial role in the mediation of the insulin secretion by modulating beta cell signaling.

The tripeptide glutathione is the most important non-enzymatic soluble intracellular antioxidant and has many metabolic functions in cellular metabolism, including attenuation of oxidative stress and inflammation^{45, 46}. The authors have demonstrated that carnosine and hannaneh peptides promotes beta cells survival and therefore represents a new and promising peptide for therapeutic and prevention strategies aimed at improving cell mass and function. In this present study, the hannaneh peptide had multiple functions including anti-DPP-4 and insulin secretion stimulator causing normal conditions in beta cells signaling and triggering blood glucose homeostasis and a balance in body weight for prevention from diabetes induced by MLDS in mice model.

In conclusion, the Leucine-glycine (Hannaneh) as a dipeptide with natural L-amino acids and carnosine may exhibit controlling effects on blood glucose homeostasis by protection of beta cell signaling and anti DPP-4 activity, and also body weight loss curbing with hannaneh, influenced by the diabetes induction. Based on the new strategies for peptides delivery and the ability of the gastrointestinal system to directly absorb the dipeptides, hannaneh and

carnosine may grasp some attraction as pharmaceutical agents to attenuate diabetes development.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare in association with this work.

ETHICAL APPROVAL

Ministry of Health and Medical Education ethics committee (number 95.93, 2015).

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Figure legends

Figure 1. The chemical structures of the studied peptides. **A:** Ala-His (carnosine), **B:** Glu-Cys-Gly (glutathione), **C:** Leu-Pro-Pro, **D:** Leu-Gly, **E:** Pro-Gly-Pro, **F:** Pro-Pro, **G:** Pro-Gly. These chemical structures were designed using Marvin Beans, 16.8.22.0 (Marvin Sketch) software for windows in a two-dimensional manner

Figure 2. The effect of the studied peptides administration on the blood glucose levels in diabetes-induced mice at days 10 (L: left) and 12 (R: right) of the experiment. **CON:** Control group (only citrate buffer injection), **DI:** Exposed to diabetes, **DM:** DI and 3% *dimethyl sulfoxide* (DMSO) as the solvent for the peptides (without a peptide), **A:** DM + Ala-His (carnosine), **B:** DM + Glu-Cys-Gly (glutathione), **C:** DM + Leu-Pro-Pro, **D:** DM + Leu-Gly, **E:** DM + Pro-Gly-Pro, **F:** DM + Pro-Pro, **G:** DM + Pro-Gly. The mean columns of each diagram with no common superscript letter differ significantly ($P < 0.05$) ($n = 10$)

Figure 3. The effect of the administration of the studied peptides on the body weight of diabetes-induced mice at the first (35.38 ± 0.27 g in all treatments), 6th and 12th days of the experiment. **CON:** Control group (only citrate buffer injection), **DI:** Exposed to diabetes, **DM:** DI and 3% *dimethyl sulfoxide* (DMSO) as the solvent for the peptides (without a

peptide), **A:** DM + Ala-His (carnosine), **B:** DM + Glu-Cys-Gly (glutathione), **C:** DM + Leu-Pro-Pro, **D:** DM + Leu-Gly, **E:** DM + Pro-Gly-Pro, **F:** DM + Pro-Pro, **G:** DM + Pro-Gly. **0:** non-significant with the first day of the experiment, **+**: significant increase ($P<0.05$), **++:** very significant increase ($P<0.01$), **-** : significant decrease ($P<0.05$), **--** : very significant decrease ($P<0.01$) (n=10)

Figure 4. The effect of peptide administration on the plasma insulin level in diabetes-induced mice on the 6th and 12th experimental days. **CON:** Control group (only citrate buffer injection), **DI:** Exposed to diabetes, **DM:** DI and 3% *dimethyl sulfoxide* (DMSO) as the solvent for the peptides (without a peptide), **A:** DM + Ala-His (carnosine), **B:** DM + Glu-Cys-Gly (glutathione), **C:** DM + Leu-Pro-Pro, **D:** DM + Leu-Gly, **E:** DM + Pro-Gly-Pro, **F:** DM + Pro-Pro, **G:** DM + Pro-Gly. The mean columns of each diagram with no common superscript letter differ significantly ($P<0.05$) (n=10)

Figure 5. The effect of peptide administration on the intracellular Akt-2 and cAMP levels in diabetes-induced mice at the end of the experiment. **CON:** Control group (only citrate buffer injection), **DI:** Exposed to diabetes, **DM:** DI and 3% *dimethyl sulfoxide* (DMSO) as the solvent for the peptides (without a peptide), **A:** DM + Ala-His (carnosine), **B:** DM + Glu-Cys-Gly (glutathione), **C:** DM + Leu-Pro-Pro, **D:** DM + Leu-Gly, **E:** DM + Pro-Gly-Pro, **F:** DM + Pro-Pro, **G:** DM + Pro-Gly. The mean columns of each diagram with no common superscript letter differ significantly ($P<0.05$) (n = 10)

Figure 6. The effect of administration of the studied peptides. **A:** Ala-His (carnosine), **B:** Glu-Cys-Gly (glutathione), **C:** Leu-Pro-Pro, **D:** Leu-Gly, **E:** Pro-Gly-Pro, **F:** Pro-Pro, and **G:** Pro-Gly compared to control (**CON**), exposed to diabetes (**DI**), and DI+ 3% DMSO (**DM**) on beta cell function assessed by immunofluorescence staining of the islets on the 12th day of the experiment (the insulin content of the beta cells is shown in green and glucagon-producing alpha cells are red, Original magnification: 1000X)

TABLE 1. Comparison of mean plasma glucose level (mg/dl) between treatments at different time points (2, 4, 6, 8, 10, and 12 days)

Treatments	Time (day)					
Time stages	2	4	6	8	10	12
CON	94.6±1.7 ^b	107.4±3.1 ^{abc}	120.3±4.2 ^{abc}	99.2±4.0 ^a	111.7±4.5 ^a	118.7±4.5 ^a
DI	111.6±2.1 ^{cd}	113.9±2.5 ^{bc}	146.4±4.5 ^{cd}	159.8±4.9 ^b	174.8±6.8 ^b	279.3±14.5 ^d
DM	104.4±2.0 ^{bcd}	122.4±2.4 ^{cd}	134.3±3.2 ^{bcd}	133.3±4.3 ^{ab}	177.3±6.5 ^b	219.1±12.1 ^c
A	101.1±1.7 ^{bc}	94.3±2.2 ^{ab}	98.0±2.8 ^a	126.1±4.0 ^{ab}	116.4±4.3 ^a	138.8±4.9 ^{ab}
B	117.9±2.3 ^d	109.7±3.1 ^{abc}	108.9±3.5 ^{ab}	134.2±4.4 ^{ab}	127.3±5.0 ^{ab}	199.6±8.1 ^{bc}
C	104.4±1.8 ^{bcd}	113.2±3.3 ^{bc}	121.0±4.3 ^{abc}	143.4±4.9 ^b	159.6±5.7 ^{ab}	190.4±7.2 ^{bc}
D	110.3±1.9 ^{bcd}	138.2±4.1 ^d	150.6±4.6 ^{cd}	145.0±4.8 ^b	112.0±4.9 ^a	146.0±5.1 ^{ab}
E	78.9±1.4 ^a	86.4±2.0 ^a	139.9±4.0 ^{bcd}	144.8±4.7 ^b	140.9±5.5 ^{ab}	172.6±8.1 ^{abc}
F	118.4±2.0 ^d	109.6±2.4 ^{abc}	130.0±4.0 ^{abcd}	127.1±3.9 ^{ab}	129.3±4.9 ^{ab}	157.9±5.7 ^{ab}
G	109.7±2.1 ^{bcd}	101.7±2.9 ^{abc}	121.4±3.1 ^{abc}	139.3±4.0 ^{ab}	132.8±4.4 ^{ab}	186.8±7.9 ^{bc}
P-Value	0.00	0.00	0.01	0.05	0.03	0.00

Means ± SD within each column with no common superscript letter differ significantly. **CON:** Control group (only citrate buffer injection), **DI:** Exposed to diabetes, **DM:** DI and 3% *dimethyl sulfoxide* (DMSO) as the solvent of the peptides (without of peptide), **A:** DM + Ala-His (Carnosine), **B:** DM + Glu-Cys-Gly (Glutathione), **C:** DM + Leu-Pro-Pro, **D:** DM + Leu-Gly, **E:** DM + Pro-Gly-Pro, **F:** DM + Pro-Pro, **G:** DM + Pro-Gly, (n =10)

TABLE 2. Comparison of mean plasma GLP-1 level and DPP-4 activity between treatments at two time points (6 and 12 days)

Parameter	GLP-1 (ng/L)	GLP-1 (ng/L)	DPP-4 (ng/ml)	DPP-4 (ng/ml)
Time stages	6	12	6	12
CON	332.5±9.1 ^{ab}	303.4±8.1 ^{abcd}	1.9±0.0 ^{ab}	1.9±0.0
DI	461.9±15.4 ^c	286.0±9.5 ^{abcd}	2.0±0.1 ^a	1.9±0.1
DM	339.1±11.6 ^{ab}	255.3±8.2 ^{ab}	1.6±0.1 ^{abcd}	1.8±0.1
A	277.8±8.9 ^a	340.5±9.4 ^{cd}	1.5±0.1 ^{bcd}	1.8±0.0
B	405.4±12.4 ^{bc}	346.7±10.0 ^d	1.6±0.1 ^{abcd}	2.1±0.1
C	350.8±11.0 ^{ab}	275.0±8.9 ^{abcd}	1.6±0.1 ^{abcd}	1.8±0.1
D	338.2±9.1 ^{ab}	249.5±8.6 ^a	1.5±0.0 ^{cd}	1.8±0.0
E	330.8±9.3 ^{ab}	262.9±9.8 ^{abc}	1.7±0.0 ^{abcd}	1.9±0.0
F	331.5±9.7 ^{ab}	235.3±9.1 ^a	1.4±0.0 ^d	1.8±0.0
G	387.2±9.6 ^{bc}	336.6±9.8 ^{bcd}	1.9±0.0 ^{abc}	1.7±0.0
P-Value	0.01	0.02	0.03	0.66

Means ± SD within each column with no common superscript letter differ significantly. **CON:** Control group (only citrate buffer injection), **DI:** Exposed to diabetes, **DM:** DI and 3% *dimethyl sulfoxide* (DMSO) as the solvent of the peptides (without of peptide), **A:** DM + Ala-His (Carnosine), **B:** DM + Glu-Cys-Gly (Glutathione), **C:** DM + Leu-Pro-Pro, **D:** DM + Leu-Gly, **E:** DM + Pro-Gly-Pro, **F:** DM + Pro-Pro, **G:** DM + Pro-Gly, (n=10)







